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<b>(54) Title:</b> AGGREGATES OF HUMAN INSULIN DERIVATIVES		
<b>(57) Abstract</b>		
<p>Water-soluble aggregates of derivatives of human insulin which have a protracted profile of action are disclosed. A new mechanism is involved in prolonging the action of the soluble insulin derivatives. This mechanism is based on the partly or fully formation of soluble aggregated forms of the derivatives, featuring a size larger than aldolase (Mw = 158 kDa) in a defined gel filtration system.</p>		

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## AGGREGATES OF HUMAN INSULIN DERIVATIVES

### Field of the invention

The present invention relates to protracted acting, water-soluble aggregates of derivatives of human insulin, derivatives of human insulin capable of forming such aggregates, pharmaceutical compositions containing them, and to the use of such aggregates in the treatment of diabetes.

### Background of the invention

Diabetes is a general term for disorders in man having excessive urine excretion as in diabetes mellitus and diabetes insipidus. Diabetes mellitus is a metabolic disorder in which the ability to utilize glucose is more or less completely lost. About 2 % of all people suffer from diabetes.

Since the introduction of insulin in the 1920's, continuous strides have been made to improve the treatment of diabetes mellitus. To help avoid extreme glycaemia levels, diabetic patients often practice multiple injection therapy, whereby insulin is administered with each meal. Many diabetic patients are treated with multiple daily insulin injections in a regimen comprising one or two daily injections of a protracted insulin to cover the basal requirement supplemented by bolus injections of a rapid acting insulin to cover the meal-related requirements.

Protracted insulin compositions are well known in the art. Thus, one main type of protracted insulin compositions comprises injectable aqueous suspensions of insulin crystals or amorphous insulin. In these compositions, the insulin compounds utilised typically are protamine insulin, zinc insulin or protamine zinc insulin.

When human or animal insulin is brought to form higher associated forms, e.g. in the presence of  $Zn^{2+}$ -ions, precipitation in the form of crystals or amorphous product is the result (Brange, Galenics of Insulin, pp. 20-27, Springer Verlag 1987). Thus, at pH 7 and using 6  $Zn^{2+}$ /hexamer of porcine insulin the result is an almost complete precipitation from solution (Grant, Biochem J. 126, 433-440, 1972). The highest soluble aggregate suggested is composed of 4 hexameric units, corresponding to a molecular weight of about 144 kDa. Blundell et al. (Diabetes 21 (Suppl. 2), 492-505, 1972) describe the soluble unit

of porcine insulin in the presence of  $Zn^{2+}$  at pH 7 as a hexamer. Early ultracentrifugation studies at pH 2 showed the insulin dimer, Mw 12 kDa, to be the prevailing species (Jeffrey, *Nature* 197, 1104-1105, 1963; Jeffrey, *Biochemistry* 5, 489-498, 1966; Jeffrey, *Biochemistry* 5, 3820-3824, 1966). Fredericq, working at pH 8 and using 0.4-0.8% (w/w)  $Zn^{2+}$  relative to insulin, reported a molecular weight of 72 kDa, corresponding to a dodecameric structure and, using 1% Zn, molecular weights of about 200-300 kDa (*Arch. Biochem Biophys.* 65, 218-228, 1956). A comprehensive review of the association states of animal insulin is found in Blundell et al. (*Adv. Protein Chem.* 26, 297-330, 1972).

Certain drawbacks are associated with the use of insulin suspensions. Thus, in order to secure an accurate dosing, the insulin particles must be suspended homogeneously by gentle shaking before a defined volume of the suspension is withdrawn from a vial or expelled from a cartridge. Also, for the storage of insulin suspensions, the temperature must be kept within more narrow limits than for insulin solutions in order to avoid lump formation or coagulation.

While it was earlier believed that protamines were non-immunogenic, it has now turned out that protamines can be immunogenic in man and that their use for medical purposes may lead to formation of antibodies (Samuel et al., *Studies on the immunogenicity of protamines in humans and experimental animals by means of a micro-complement fixation test*, *Clin. Exp. Immunol.* 33, pp. 252-260 (1978)).

Also, evidence has been found that the protamine-insulin complex is itself immunogenic (Kurtz et al., *Circulating IgG antibody to protamine in patients treated with protamine-insulins*, *Diabetologica* 25, pp. 322-324 (1983)). Therefore, with some patients the use of protracted insulin compositions containing protamines must be avoided.

Another type of protracted insulin compositions are solutions having a pH value below physiological pH from which the insulin will precipitate because of the rise in the pH value when the solution is injected. A drawback is that the solid particles of the insulin act as a local irritant causing inflammation of the tissue at the site of injection.

WO 91/12817 (Novo Nordisk A/S) discloses protracted, soluble insulin compositions comprising insulin complexes of cobalt(III). The protraction of these complexes is only intermediate and the bioavailability is reduced.

Soluble insulin derivatives containing lipophilic substituents linked to the  $\epsilon$ -amino group of

a lysine residue in any of the positions B26 to B30 have been described in e.g. WO 95/07931 (Novo Nordisk A/S), WO 96/00107 (Novo Nordisk A/S) and WO 97/31022 (Novo Nordisk A/S). Such derivatives have a protracted action after subcutaneous injection as compared to soluble human insulin, and this protracted action has been explained by a reversible binding to albumin in subcutis, blood and peripheral tissue (Markussen, Diabetologia 39, 281-288, 1996; Kurzhals, Biochem J. 312, 725-731, 1995; Kurzhals, J. Pharm Sciences 85, 304-308, 1996; and Whittingham, Biochemistry 36, 2826-2831, 1997).

However, we have now discovered a new mechanism of prolonging the action of some of the soluble insulin derivatives. The new mechanism is based on the partly or fully formation of soluble aggregated forms of the derivatives, featuring a size larger than aldolase (Mw = 158 kDa) in a defined gel filtration system.

### Brief Description of the Drawings

Fig. 1. Calibration curve of  $K_{AV}$  values versus molecular weight in the gel filtration system using a column of Sephacryl® S-300 HR in an aqueous neutral eluent comprising 125 mM sodium chloride and 20 mM sodium phosphate at pH 7.4. A near linear relation between  $K_{AV}$  and the logarithm of the molecular weight is apparent. The standards are shown in Table 1.

Fig. 2. Gel filtration of Lys<sup>B29</sup>(N<sup>ε</sup> ω-carboxyheptadecanoyl) des(B30) human insulin having 0, 2 and 3 Zn<sup>2+</sup>/hexamer, respectively, using a column of Sephacryl® S-300 HR in an aqueous neutral eluent comprising 125 mM sodium chloride and 20 mM sodium phosphate at pH 7.4, demonstrating the importance of Zn<sup>2+</sup> for the formation of aggregates for this derivative. A column of 28 x 1 cm is eluted at a rate of 15 ml/h. Insulin derivatives were injected (200 μl) as a standard preparation comprising 600 μM derivative, 0, 2 or 3 Zn<sup>2+</sup>/6 molecules of insulin, 20 mM NaCl, 16 mM phenol, 16 mM m-cresol, 7 mM sodium phosphate at pH 7.5.

Fig. 3. Gel filtration of Lys<sup>B29</sup>(N<sup>ε</sup> ω-carboxyheptadecanoyl) des(B30) human insulin having 3 Zn<sup>2+</sup>/hexamer using a column of Sephacryl® S-300 HR in an aqueous neutral eluent comprising 5 mM sodium phosphate buffer pH 7.5, 10 mM sodium chloride, 16 mM phenol, 16 mM m-cresol and 1.6% (w/v) glycerol. A comparison to Fig. 2 elucidates the

importance of the sodium chloride concentration for the formation of aggregates of this derivative.

Fig.4. Scheme of the synthesis of the conjugated ligands.

## Description of the Invention

- 5 The expression "insulin derivative" as used herein (and related expressions) refers to human insulin or an analogue thereof in which at least one organic substituent is bound to one or more of the amino acids.

- By "analogue of human insulin" as used herein (and related expressions) is meant human insulin in which one or more amino acids have been deleted and/or replaced by other  
10 amino acids, including non-codeable amino acids, or human insulin comprising additional amino acids, i.e. more than 51 amino acids.

- The present invention is based on the discovery of a new aggregated and soluble form of insulin derivatives. The new, soluble aggregated form of insulin derivatives dissociates slowly after subcutaneous injection, making them suitable for a long-acting insulin  
15 preparation, the advantage being that the preparation contains no precipitate. The advantages of soluble rather than suspension preparations are higher precision in dosing, avoidance of shaking of the vial or pen, allowance for a thinner needle meaning less pain during injection, easier filling of vials or cartridge and avoidance of a ball in the cartridge used to suspend the precipitate in the absence of air.

- 20 More specifically, the present invention relates to a water-soluble aggregate of insulin derivatives, characterised by having a size larger than aldolase, preferably larger than ferritin, as determined by a gel filtration system as specified herein.

- The aggregate according to the invention preferably has an apparent volume corresponding to a  $K_{AV}$  value of less than 0.32, preferably less than 0.20, as determined by gel  
25 filtration using a Sephacryl® S-300 HR gel, or a  $K_{AV}$  value of less than 0.50, preferably less than 0.40, as determined by gel filtration using a Superose® 6HR gel.

The aggregate is preferably soluble at a pH in the range of 6.8 to 8.5.

The new aggregated form can be observed for insulin derivatives under conditions where

the hexameric unit is known to exist for most insulins. Thus, in a preferred embodiment, the aggregated form is composed of hexameric subunits, preferably of at least 4, more preferably 5 to 50, still more preferably 5 to 200, hexameric subunits. Any hexameric subunit of the aggregated forms of this invention may have any of the known R<sub>6</sub>, R<sub>3</sub>T<sub>3</sub>, or T<sub>6</sub> structures (Kaarsholm, Biochemistry 28, 4427-4435, 1989).

Substances like Zn<sup>2+</sup> and phenolic compounds known to stabilise the hexameric unit are also found to stabilise the new aggregated form of some insulin derivatives. The building blocks forming the aggregates may be the hexameric units known from the X-ray crystallographic determined structure of insulin (Blundell, Diabetes 21 (Suppl. 2), 492-505, 1972). Ions like Zn<sup>2+</sup>, known to stabilise the hexameric unit as 2 or 4 Zn<sup>2+</sup>/hexamer complexes (Blundell, Diabetes 21 (Suppl. 2), 492-505, 1972), are essential for the formation of aggregates for some derivatives, like for Lys<sup>B29</sup>(N<sup>ε</sup> ω-carboxyheptadecanoyl) des(B30) human insulin. Fig. 2 shows gel filtration of Lys<sup>B29</sup>(N<sup>ε</sup> ω-carboxyheptadecanoyl) des(B30) human insulin in the system described herein of preparations containing 0, 2, and 3 Zn<sup>2+</sup>/hexamer, respectively. In the absence of Zn<sup>2+</sup> aggregates are not formed, the elution position indicating the presence of a monomer or dimer. Thus, the aggregate according to invention preferably comprises at least 2 zinc ions, more preferably 2 to 5 zinc ions, still more preferably 2 to 3 zinc ions, per 6 molecules of insulin derivative. Moreover, the aggregate advantageously comprises at least 3 molecules of a phenolic compound per 6 molecules of insulin derivative. In the central cavity of the 2 Zn<sup>2+</sup>/hexamer structure 6 residues of Glu<sup>B13</sup> provide binding sites for up to 3 Ca<sup>2+</sup> ions (Sudmeier et al., Science 212, 560-562, 1981). Thus, addition of Ca<sup>2+</sup> ions stabilises the hexamer and may be added to the pharmaceutical formulations, on the condition that the insulin derivative remains in solution.

The disappearance half-time of the aggregate of the invention after subcutaneous injection in humans is preferably as long as or longer than that of a human insulin NPH preparation.

In a particularly preferred embodiment of the present invention, the aggregate is composed of insulin derivatives which have an albumin binding which is lower than that of Lys<sup>B29</sup>(N<sup>ε</sup> tetradecanoyl) des(B30) human insulin.

The preferred primary structures of insulin derivatives to be employed in the present invention are those in which:

- a) the residues B24-B30 of the B-chain of the insulin derivative is the sequence Phe-X-X-X-X-X-X, where each X independently represents any codable amino acid or a deletion;
- b) the residues B25-B30 of the B-chain of the insulin derivative is the sequence Phe-X-X-X-X-X, where each X independently represents any codable amino acid or a deletion;
- 5 c) the residues B26-B30 of the B-chain of the insulin derivative is the sequence Tyr-X-X-X-X, where each X independently represents any codable amino acid or a deletion;
- d) the residues B27-B30 of the B-chain of the insulin derivative is the sequence Thr-X-X-X, where each X independently represents any codable amino acid or a deletion;
- e) the residues B28-B30 of the B-chain of the insulin derivative is the sequence Pro-X-X, where each X independently represents any codable amino acid or a deletion; or
- 10 f) the residues B29-B30 of the B-chain of the insulin derivative is the sequence Lys-X, where X represents any codable amino acid or a deletion;

provided that the insulin derivative exhibits a potency of at least 5%, e.g. as assessed by the free fat cell assay or by affinity to the insulin receptor.

- 15 The substituent at the lysine residue of the insulin derivative of the aggregate according to the invention is preferably a lipophilic group containing from 6 to 40 carbon atoms. More preferred are substituents which are acyl groups having from 6 to 40, preferably 12 to 36, carbon atoms.

The most preferred lipophilic substituents in the form of acyl groups are the following: CH<sub>3</sub>-(CH<sub>2</sub>)<sub>n</sub>-CO-, (COOH)-(CH<sub>2</sub>)<sub>n</sub>-CO-, (NH<sub>2</sub>-CO)-(CH<sub>2</sub>)<sub>n</sub>-CO-, HO-(CH<sub>2</sub>)<sub>n</sub>-CO-, where 4 ≤ n ≤ 38.

20 In another preferred embodiment the lipophilic substituent is 5-α lithocholic acid or 5-β lithocholic acid.

In another preferred embodiment the lipophilic substituent is 5-α or 5-β isomers of cholic acid, hyocholic acid, deoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid, hyodeoxycholic acid or cholanic acid.

25 In another preferred embodiment the lipophilic substituent is fusidic acid, a fusidic acid derivative or glycyrrhetic acid.



In yet another preferred embodiment the lipophilic substituent is connected to a lysine residue using an amino acid linker. According to this embodiment the lipophilic substituent is advantageously connected to a lysine residue via a  $\gamma$ - or an  $\alpha$ -glutamyl linker, or via a  $\beta$ - or an  $\alpha$ -aspartyl linker, or via an  $\alpha$ -amido- $\gamma$ -glutamyl linker, or via an  $\alpha$ -amido- $\beta$ -aspartyl linker.

The present invention furthermore provides novel insulin derivatives capable of forming aggregates. These insulin derivatives may be provided in the form of aggregates in pharmaceutical preparations or, alternatively, they may be provided in a non-aggregated form in pharmaceutical preparations, in which case the aggregates form after subcutaneous injection of said preparations.

Accordingly, the present invention furthermore is concerned with pharmaceutical preparations comprising an aggregate of insulin derivatives or non-aggregated insulin derivatives which form aggregates after subcutaneous injection.

Preferably, the pharmaceutical preparation according to the present invention comprises aggregates, a substantial fraction of which (preferably more than 75%) has a larger size than aldolase as determined by gel filtration using the medium of the preparation as eluent.

In another embodiment, a pharmaceutical preparation comprising both aggregating and rapid acting insulin analogues, the latter preferably being human insulin or one of the insulin analogues Asp<sup>B28</sup> human insulin, Lys<sup>B28</sup>Pro<sup>B29</sup> human insulin or des(B30) human insulin, is provided. Such a preparation will provide both a rapid onset of action as well as a prolonged action profile.

In this embodiment, the pharmaceutical preparation preferably comprises aggregating insulin and rapid acting insulin in a molar ratio of 90:10 to 10:90.

The slow dissociation of the aggregated forms may be further slowed down in vivo by the addition of physiological acceptable agents that increase the viscosity of the pharmaceutical preparation. Thus, the pharmaceutical preparation according to the invention may furthermore comprise an agent which increases the viscosity, preferably polyethylene glycol, polypropylene glycol, copolymers thereof, dextrans and/or polylactides.

The pharmaceutical preparation preferably further comprises a buffer substance, such as

a TRIS, phosphate, glycine or glycyglycine (or another zwitterionic substance) buffer, an isotonicity agent, such as NaCl, glycerol, mannitol and/or lactose, and phenol and/or m-cresol as preservatives. Among the auxiliary substances of a pharmaceutical preparation the sodium chloride, used as isotonic agent, and the phenol, used for preservation, are  
5 particular important by promoting the aggregation in the preparation and thereby effectively prolong the time of disappearance from the site of injection. The pharmaceutical preparation according to the invention preferably comprises  $\text{Na}^+$  ions in a concentration of 10 to 150 mM.

The most preferred pharmaceutical preparation is a preparation containing 0.1-2 mM of an  
10 insulin derivative according to the present invention, 0.3-0.9% Zn (w/w relative to insulin derivative), and phenolic compounds like phenol or m-cresol or mixtures hereof in a total concentration of 5-50 mM, and  $\text{Na}^+$  ions in a concentration of 10 mM to 150 mM

The present invention furthermore relates to a method of treating diabetes mellitus comprising administering to a person in need of such treatment an effective amount of water-soluble  
15 aggregates of insulin derivatives according to the invention or effective amount an insulin derivative according to the invention, capable of forming water-soluble aggregates upon subcutaneous injection.

The insulin derivatives of the invention can be prepared by the general methods disclosed in WO 95/07931 (Novo Nordisk A/S), WO 96/00107 (Novo Nordisk A/S), WO 97/31022  
20 (Novo Nordisk A/S), PCT application No. DK97/00296 (Novo Nordisk A/S), EP 511 600 (Kurakay Co. Ltd.) and EP 712 862 (Eli Lilly). The derivatives listed in Table 2 have been prepared by selective acylation of the  $\epsilon$ -amino group of  $\text{Lys}^{B29}$  of des(B30) human insulin by the ligands activated in the form of the respective N-hydroxysuccinimide esters. The conjugated ligands can be prepared using conventional peptide chemistry (Fig. 4).

Some of the derivatives listed in the aforementioned patent applications, and described in the publications of Markussen, Diabetologia 39, 281-288, 1996; Kurzhals, Biochem J. 312, 725-731, 1995; Kurzhals, J. Pharm Sciences 85, 304-308, 1996; and Whittingham, Biochemistry 36, 2826-2831, 1997 as being protracted due to the albumin binding mechanism, do also possess the ability to form high molecular weight soluble aggregates in  
25 accordance with the present invention.  $\text{Lys}^{B29}(\text{N}^\epsilon \text{ lithocholyl-}\gamma\text{-Glu-})$  des(B30) human insulin from WO 95/07931 and  $\text{Lys}^{B29}(\text{N}^\epsilon \omega\text{-carboxyheptadecanoyl-})$  des(B30) human insulin from WO 97/31022 are examples of insulin derivatives capable of forming high  
30

molecular weight soluble aggregates at neutral pH. There is selectivity between the lipophilic substituents in their ability to induce formation of aggregates. Thus, of the two isomers, Lys<sup>B29</sup>(N<sup>ε</sup> lithocholyl-γ-Glu-) des(B30) human insulin and Lys<sup>B29</sup>(N<sup>ε</sup> lithocholyl-α-Glu-) des(B30) human insulin, only the first forms aggregates in the formulation used, see Table 1.

#### DETERMINATION OF AGGREGATE FORMATION

The aggregated form is demonstrated by gel filtration using a gel with an exclusion limit of 1,500 kDa for globular proteins and 400 kDa for linear dextrans. A pH neutral aqueous buffer system is used in the gel filtration and the insulin derivatives in the aggregated state are applied to the column in the form of a pharmaceutical preparation at a concentration of 600 nmol insulin/ml. The aggregated states of the insulin derivatives elute before aldolase, which has a molecular weight of 158 kDa.

The gel filtration experiment using the conditions prescribed in this section is the direct physico-chemical method to reveal the potential aggregate formation property of an insulin derivative. Disappearance after subcutaneous injection in pigs reflects the combination of the albumin binding and polymer formation properties of the insulin derivative, besides a variety of biological factors.

The formation of high molecular weight soluble aggregates is demonstrated by gel filtration using a column of Sephacryl<sup>®</sup> S-300 HR in an aqueous neutral eluent comprising 125 mM sodium chloride and 20 mM sodium phosphate at pH 7.4. This buffer system was chosen to mimic the ionic strength and pH of the tissue, in order to be able to detect derivatives aggregated under conditions similar to those after the subcutaneous injection. Obviously, in other buffer systems having lower concentration of sodium chloride or a lower or higher pH value the derivatives may not appear in the aggregated state. However, when the actual state of aggregation in a pharmaceutical preparation is to be assessed, the medium of the preparation, exclusive the Zn<sup>2+</sup> which is insulin bound, is used as the eluent for the gel filtration.

A column of 28 x 1 cm is eluted at a rate of 15 ml/h. Insulin derivatives were injected (200 μl) as a standard formulation comprising 600 μM derivative, 200 or 300 μM Zn<sup>2+</sup>, 20 mM NaCl (or varied), 16 mM phenol, 16 mM m-cresol, 7 mM sodium phosphate at pH 7.5.

Exclusion limit of Sephacryl<sup>®</sup> S-300 HR is stated by the manufacturer, Pharmacia, as a

molecular weight of 1,500 kDa for globular proteins and 400 kDa for linear dextrans. In practice the elution of solutes of different size is characterised by the available volume as  $K_{AV}$  values:

$$K_{AV} = (V_E - V_0)/(V_T - V_0)$$

- 5 where  $V_E$  is elution volume,  $V_0$  is void volume, e.g. elution volume of blue dextran,  $V_T$  is total volume. Thus, the  $K_{AV}$  value is independent of column dimension. In this system aldolase (Mw 158 kDa) elutes at about a  $K_{AV}$  of 0.32, albumin (Mw of 67 kDa) at about a  $K_{AV}$  of 0.38, and the monomeric form of insulin (Mw of 6 kDa) with a  $K_{AV}$  of about 0.71. The calibration of the column using a series of molecular weight standards shows a near  
10 linear relation between  $K_{AV}$  and the logarithm of the molecular weight, see Fig. 1.

TABLE 1.

- $K_{AV}$  values, albumin binding constants and disappearance half-times for associating insulin derivatives larger than aldolase (Mw 158 kDa), non-associating insulin derivatives smaller than aldolase and standards used as markers of molecular size. Albumin binding  
15 constants and disappearance half times in pigs have been normalised using Lys<sup>B29</sup>(N<sup>ε</sup> tetradecanoyl) des(B30) human insulin as the reference compound. Disappearance  $T_{50\%}$  for NPH insulin in pigs have been measured to 10.5 h (Markussen et al. 1996).

Compounds		K <sub>AV</sub>	Albumin binding K <sub>ass.</sub> (mol/l) <sup>-1</sup>	Disappearance T <sub>50%</sub> , h
<i>Associating derivatives of human insulin forming aggregates larger than aldolase. **</i>				
5	LysB29(N <sup>ε</sup> lithocholyl-γ-Glu-) des(B30)	0.04*	0.3x10 <sup>5</sup>	22.8
	LysB29(N <sup>ε</sup> ω-carboxyheptadecanoyl) des(B30)	0.05	25x10 <sup>5</sup>	18.7
	LysB29(N <sup>ε</sup> ω-carboxynonadecanoyl) des(B30)	0.04	36x10 <sup>5</sup>	21.9
	LysB29(N <sup>ε</sup> cholesteryloxycarbonyl)	0.00		
<i>Non-associating derivatives of human insulin forming aggregates smaller than aldolase. **</i>				
	Human insulin***	0.61	0	(2)
	Human insulin (Zinc free)	0.72		
15	LysB29(N <sup>ε</sup> lithocholyl (Zinc free)	0.74		
	LysB29(N <sup>ε</sup> decanoyl) ***	0.67	0.06x10 <sup>5</sup>	5.1
	LysB29(N <sup>ε</sup> tetradecanoyl) des(B30)	0.51	1.0x10 <sup>5</sup>	14.3
	LysB29(N <sup>ε</sup> lithocholyl-α-Glu-) des(B30)	0.53	0.3x10 <sup>5</sup>	11.8
<i>Standards. ****</i>				
20	B9Asp, B27Glu human insulin (monomeric, Mw 6 kDa)	0.71	0	(1)
	Ribonuclease (Mw 13.7 kDa)	0.63		
	Albumin (Mw 67 kDa)	0.38		
	Aldolase (Mw 158 kDa)	0.32		
	Catalase (Mw 232 kDa)	0.30		
25	Ferritin (Mw 440 kDa)	0.19		
	Thyroglobulin (Mw 669 kDa)	0.08		

\*75% of the derivatives eluted in the main peak, and 25% in the position of the monomer or dimer.

\*\*Applied 200 μl sample as a pharmaceutical preparation comprising 600 μM of derivative, 200 μM Zn<sup>2+</sup>, 0-20 mM sodium chloride, 7 mM sodium phosphate, 16 mM phenol, 16 mM m-cresol, 1.6 % glycerol and pH of 7.5.

\*\*\*Same as \*\* but 300  $\mu\text{M}$   $\text{Zn}^{2+}$ .

\*\*\*\*Standards applied dissolved in water.

Examples of insulin derivatives capable of forming soluble high molecular weight aggregates and having a protracted action based primarily on this property are Lys<sup>B29</sup>(N<sup>ε</sup> lithocholyl- $\gamma$ -Glu-) des(B30) human insulin, see Table 1. Notably, the ratio between disappearance half time and albumin binding constant is high for this class of compounds. Examples of insulin derivatives incapable of forming soluble high molecular weight aggregates but having a protracted action based on the albumin binding property are Lys<sup>B29</sup>(N<sup>ε</sup> lithocholyl- $\alpha$ -Glu-) des(B30) human insulin and Lys<sup>B29</sup>(N<sup>ε</sup>-tetradecanoyl-) des(B30) human insulin, see Table 1. Notably, the ratio between disappearance half time/albumin binding constant is low for this class of compounds.

In WO 97/31022 a pharmaceutical preparation of Lys<sup>B29</sup>(N<sup>ε</sup>- $\omega$ -carboxyheptadecanoyl) des(B30) human insulin has been formulated comprising 600 nmol/ml of derivative, 5 mM sodium phosphate buffer pH 7.5, 10 mM sodium chloride, 16 mM phenol, 16 mM m-cresol, 2-3  $\text{Zn}^{2+}$ /hexamer and 1.6%(w/v) glycerol. In order to establish the degree of aggregation in this formulation a gel filtration was performed using the same column as described above but using the medium of the preparation as the eluent. The  $\text{Zn}^{2+}$  is mostly insulin bound and is therefore not considered a constituent of the medium. Since the eluent contains phenolic substances the concentration of derivative in the fractions is monitored by HPLC, see Fig. 3. The  $K_{AV}$  value of about 0.45 indicates that hexameric or dodecameric units are the prevailing species in the preparation, i.e. no high molecular weight aggregates of insulin derivatives was present in this published formulation.

An alternative method to measure the capability of insulin derivatives of forming soluble high molecular weight aggregates was developed, suitable for HPLC equipment. The column dimensions, injection volume, and flow rate correspond to the first method, whereas the temperature is increased to 37 °C and the phosphate buffer is changed to trishydroxymethylaminomethan hydrochloride and additional sodium chloride. The aggregated state of insulin is defined to elute before the gel filtration standard aldolase like in the first method.

$K_{AV}$ -values are shown for two levels of zinc in Table 2. Compared to the reference, Lys<sup>B29</sup>(N<sup>ε</sup> tetradecanoyl-) des(B30) insulin, a long disappearance time from a subcutaneous depot is correlated with a tendency of the insulin derivative to form large aggregates.

Table 2. Aggregate formation of insulin derivatives measured by gel filtration method 2.

Compounds	K <sub>AV</sub> (Superose 6HR) <sup>2)</sup>		Albumin binding K <sub>ass.</sub> ( 10 <sup>5</sup> M <sup>-1</sup> )	Disappearance in pigs <sup>1)</sup> , T <sub>50%</sub> .(h)
	2Zn <sup>2+</sup> /6 ins	3 Zn <sup>2+</sup> /6 ins		
LysB <sup>29</sup> (N <sup>ε</sup> -lithocholoyl-γ-Glu-) des(B30) HI	0.00	-0.01	0.33	22.8
LysB <sup>29</sup> (N <sup>ε</sup> -deoxycholoyl-γ-Glu-) des(B30) HI	0.20	0.07	0.03	13.9
LysB <sup>29</sup> (N <sup>ε</sup> -lithocholoyl-α-amido-γ-Glu-) des(B30) HI	-0.02	0.00	0.23	>34
LysB <sup>29</sup> (N <sup>ε</sup> -lithocholoyl-β-Asp-) des(B30) HI	0.18	0.11	n.d.	n.d.
LysB <sup>29</sup> (N <sup>ε</sup> -lithocholoyl-β-Ala-) des(B30) HI	0.00	0.13	n.d.	n.d.
LysB <sup>29</sup> (N <sup>ε</sup> -lithocholoyl-γ-aminobutanoyl-) des(B30) HI	0.06	0.00	n.d.	n.d.
LysB <sup>29</sup> (N <sup>ε</sup> -lithocholoyl-) des(B30) HI	-0.01	0.23	0.38	>34
LysB <sup>29</sup> (N <sup>ε</sup> -dehydrolithocholoyl-) des(B30) HI	0.05	0.03	0.26	>34
LysB <sup>29</sup> (N <sup>ε</sup> -cholanoyl-) des(B30) HI	0.40	0.17	0.48	20.1
LysB <sup>29</sup> (N <sup>ε</sup> -hexadecanoyl-α-	0.38	0.41	0.56	15.3

amido- $\gamma$ -Glu-) des(B30) HI				
Asp <sup>A21</sup> Lys <sup>B29</sup> (N <sup>ε</sup> -tetradecanoyl-) des(B30) HI	0.55	0.46	0.97	16.4
Lys <sup>B29</sup> (N <sup>ε</sup> -tetradecanoyl-) des(B30) HI	0.58	0.56	1.00	14.3
Human insulin, (HI)	0.64	0.64	-	2
<b>Standards:</b>				
Asp <sup>B9</sup> Glu <sup>B27</sup> HI (monomeric, Mw 6 kDa)	0.73			
Ribonuclease (Mw 13.7 kDa)	0.72			
Ovalbumin (Mw 43 kDa)	0.58			
Aldolase (Mw 158 kDa)	0.50			
Ferritin (Mw 440 kDa)	0.40			
Thyroglobulin (Mw 669 kDa)	0.28			

1) Normalised to Lys<sup>B29</sup>(N<sup>ε</sup>-tetradecanoyl-) des(B30) human insulin (T<sub>50%</sub>=14.3 h)

2) Superose 6 HR 10/30 (Pharmacia Biotech) is eluted at 37 °C by sodium chloride 140 mM, trishydroxymethylaminomethan 10 mM, sodium azide 0.02 %, and hydrochloric acid added to pH 7.4. A run time time of 90 min. (0.25 ml/min.) is followed by a washing period of 150 min. (0.5 ml/min.). The injection volume was 200  $\mu$ l.



## Claims

1. A water-soluble aggregate of insulin derivatives, characterised by having a size larger than aldolase as determined in a gel filtration system as specified above.
2. An aggregate according to claim 1, characterised by having a size larger than ferritin  
5 as determined in a gel filtration system as specified above.
3. An aggregate according to claim 1 having an apparent volume corresponding to a  $K_{AV}$  value of less than 0.32, preferably less than 0.20, as determined by gel filtration using a Sephacryl® S-300 HR gel.
4. An aggregate according to claim 1 having an apparent volume corresponding to a  
10  $K_{AV}$  value of less than 0.50, preferably less than 0.40, as determined by gel filtration using a Superose® 6HR gel.
5. An aggregate according to any one of claims 1 to 4, characterised by being soluble at a pH in the range of 6.8 to 8.5.
6. An aggregate according to any one of claims 1 to 5, composed essentially of  
15 hexameric subunits of insulin derivatives.
7. An aggregate according to claim 6, composed of at least 4, preferably 5 to 50, more preferably 5 to 200 aggregated hexameric subunits.
8. An aggregate according to any one of claims 1 to 7, comprising at least 2 zinc ions,  
20 preferably 2 to 5 zinc ions, more preferably 2 to 3 zinc ions, per 6 molecules of insulin derivative.
9. An aggregate according to any one of claims 1 to 8, comprising at least 3 molecules of a phenolic compound per 6 molecules of insulin derivative.
10. An aggregate according to any one of claims 1 to 9, having a disappearance half-  
25 time after subcutaneous injection in humans as long as or longer than that of a human insulin NPH preparation.
11. An aggregate according to any one of claims 1 to 10, in which the insulin derivative has an albumin binding which is lower than that of Lys<sup>B29</sup>(N<sup>ε</sup>-tetradecanoyl) des(B30)

human insulin.

- 5      12. An aggregate according to any one of claims 1 to 11, in which the residues B24-B30 of the B-chain of the insulin derivative is the sequence Phe-X-X-X-X-X, where each X independently represents any amino acid or a deletion, at least one X being a N $\epsilon$ -substituted lysine residue.
13. An aggregate according to any one of claims 1 to 12, in which the residues B25-B30 of the B-chain of the insulin derivative is the sequence Phe-X-X-X-X-X, where each X independently represents any amino acid or a deletion, at least one X being a N $\epsilon$ -substituted lysine residue.
- 10      14. An aggregate according to any one of claims 1 to 13, in which the residues B26-B30 of the B-chain of the insulin derivative is the sequence Tyr-X-X-X-X, where each X independently represents any amino acid or a deletion, at least one X being a N $\epsilon$ -substituted lysine residue.
- 15      15. An aggregate according to any one of claims 1 to 14, in which the residues B27-B30 of the B-chain of the insulin derivative is the sequence Thr-X-X-X, where each X independently represents any amino acid or a deletion, at least one X being a N $\epsilon$ -substituted lysine residue.
- 20      16. An aggregate according to any one of claims 1 to 15, in which the residues B28-B30 of the B-chain of the insulin derivative is the sequence Pro-X-X, where each X independently represents any amino acid or a deletion, at least one X being a N $\epsilon$ -substituted lysine residue.
17. An aggregate according to any one of claims 1 to 16, in which the residues B29-B30 of the B-chain of the insulin derivative is the sequence Lys-X, where X represents any amino acid or a deletion.
- 25      18. An aggregate according to any one of claims 12 to 17, in which the substituent at the lysine residue is a lipophilic group containing from 6 to 40 carbon atoms.
19. An aggregate according to claim 18, in which the substituent is an acyl group having from 6 to 40, preferably 12 to 36, carbon atoms.

20. An aggregate according to claim 19, in which the acyl group is  $\text{CH}_3-(\text{CH}_2)_n-\text{CO}-$ , where  $4 \leq n \leq 38$ .
21. An aggregate according to claim 19, in which the acyl group is  $(\text{COOH})-(\text{CH}_2)_n-\text{CO}-$ , where  $4 \leq n \leq 38$ .
- 5 22. An aggregate according to claim 19, in which the acyl group is  $(\text{NH}_2-\text{CO})-(\text{CH}_2)_n-\text{CO}-$ , where  $4 \leq n \leq 38$ .
23. An aggregate according to claim 19, in which the acyl group is  $\text{HO}-(\text{CH}_2)_n-\text{CO}-$ , where  $4 \leq n \leq 38$ .
- 10 24. An aggregate according to claim 19, in which the lipophilic substituent is 5- $\alpha$  lithocholic acid or 5- $\beta$  lithocholic acid..
25. An aggregate according to claim 19, in which the lipophilic substituent is 5- $\alpha$  or 5- $\beta$  isomers of cholic acid, hyocholic acid, deoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid, hyodeoxycholic acid or cholanilic acid.
- 15 26. An aggregate according to claim 19, in which the lipophilic substituent is a 5- $\alpha$  or 5- $\beta$  isomer of dehydrolithocholic acid.
27. An aggregate according to claim 19, in which the lipophilic substituent is fusidic acid, a fusidic acid derivative or glycyrrhetic acid.
28. An aggregate according to claims 19-27, in which the acyl group is linked to the lysine residue using an amino acid as linker.
- 20 29. An aggregate according to claim 28, in which the amino acid link is  $\alpha$ -glutamyl or  $\gamma$ -glutamyl bonded or  $\beta$ - or  $\alpha$ -aspartyl bonded.
30. An aggregate according to claim 28, in which the amino acid link is  $\gamma$ -aminobutanoyl bonded,  $\beta$ -alanyl bonded,  $\alpha$ -amido- $\gamma$ -glutamyl bonded, or  $\alpha$ -amido- $\beta$ -aspartyl bonded.
- 25 31. A novel insulin derivative capable of forming aggregates according to any one of the preceding claims.

32. A pharmaceutical preparation comprising an aggregate of insulin derivatives according to any one of claims 1 to 30.
- 5 33. A pharmaceutical preparation according to claim 32, comprising aggregates according to any one of claims 1 to 30, a substantial fraction of which, preferably more than 75%, has a larger size than aldolase as determined by gel filtration using the medium of the preparation as eluent.
34. A pharmaceutical preparation comprising both aggregating insulin derivatives and rapid acting insulin analogues, the latter preferably being human insulin or Asp<sup>B28</sup> human insulin, Lys<sup>B28</sup>Pro<sup>B29</sup> human insulin or des(B30) human insulin.
- 10 35. A pharmaceutical preparation according to claim 34, in which the molar ratio of aggregating insulin to rapid acting insulin is 90:10 to 10:90.
36. A pharmaceutical preparation according to any one of claims 32 to 35, comprising an agent which increases the viscosity, preferably polyethylene glycol, polypropylene glycol, copolymers thereof, dextrans and/or polylactides.
- 15 37. A pharmaceutical preparation according to any one of claims 32 to 36, comprising a buffer substance, preferably a TRIS, phosphate or glycine buffer.
38. A pharmaceutical preparation according to any one of claims 32 to 37, comprising a zwitterionic buffer substance, preferably glycylglycine.
- 20 39. A pharmaceutical preparation according to any one of claims 32 to 38, comprising an isotonic agent, preferably NaCl, glycerol, mannitol and/or lactose.
40. A pharmaceutical preparation according to any one of claims 32 to 39, comprising Na<sup>+</sup> ions in a concentration of 10 to 150 mM.
41. A pharmaceutical preparation according to any one of claims 32 to 40, comprising phenol and/or m-cresol as preservatives.
- 25 42. A pharmaceutical preparation containing 0.1-2 mM of an insulin derivative according to claim 31, 0.3-0.9% Zn (w/w relative to insulin derivative), and phenolic compounds like phenol or m-cresol or mixtures hereof in a total concentration of 5-50 mM, and Na<sup>+</sup> ions in a concentration of 10 mM to 150 mM.

43. A method of treating diabetes mellitus comprising administering to a person in need of such treatment an effective amount of water-soluble aggregates of insulin derivatives according to any one of the claims 1 to 30.
- 5 44. A method of treating diabetes mellitus comprising administering to a person in need of such treatment an effective amount an insulin derivative according to claim 31, capable of forming water-soluble aggregates upon subcutaneous injection.

Fig. 1

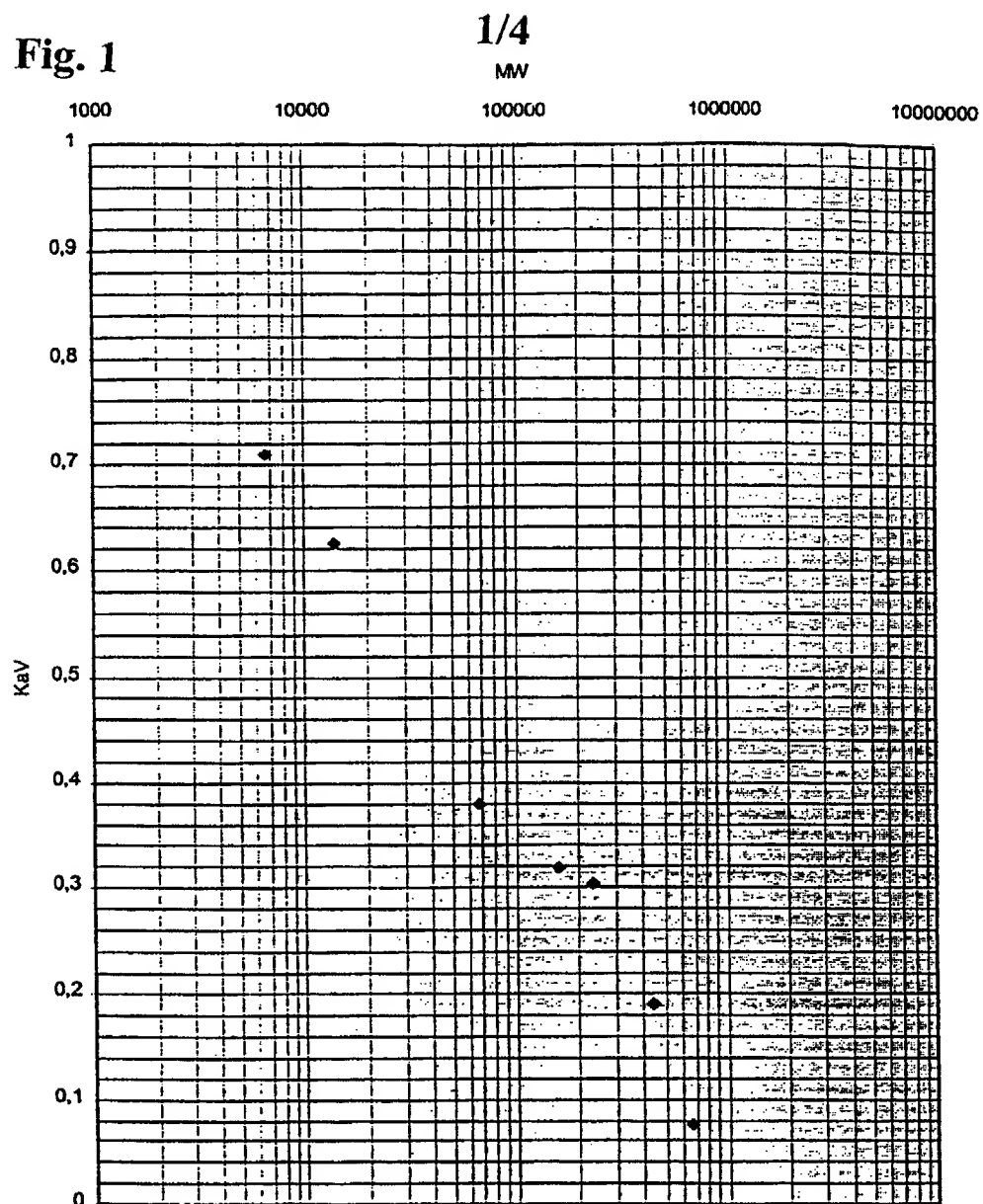


Fig. 2

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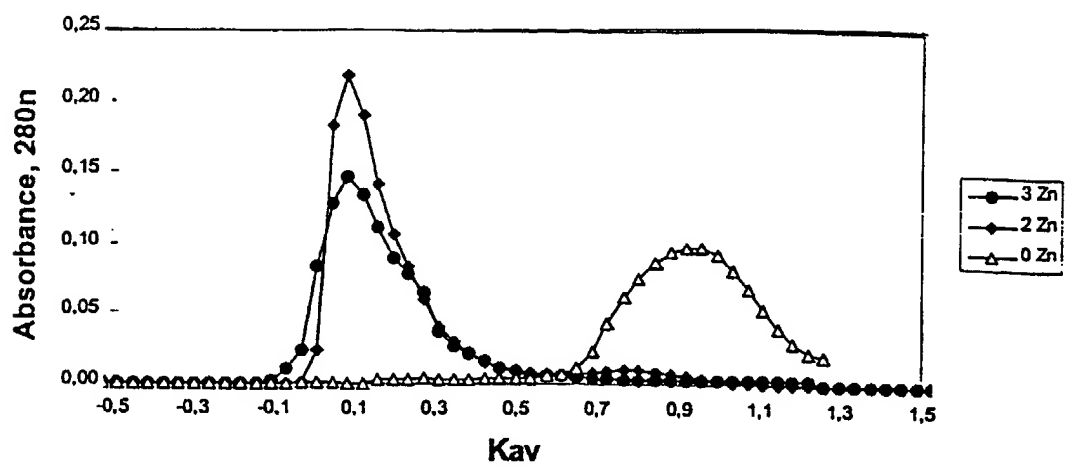


Fig. 3

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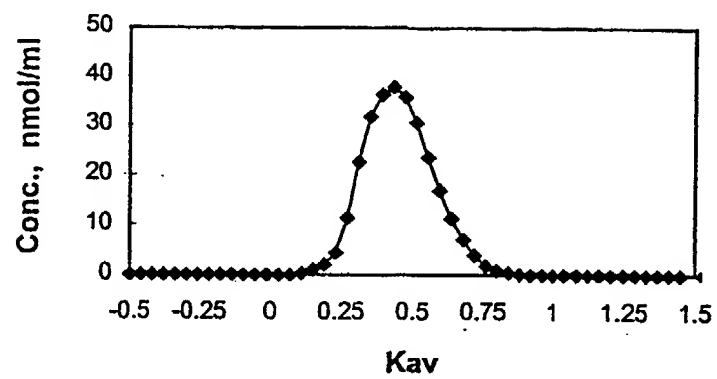
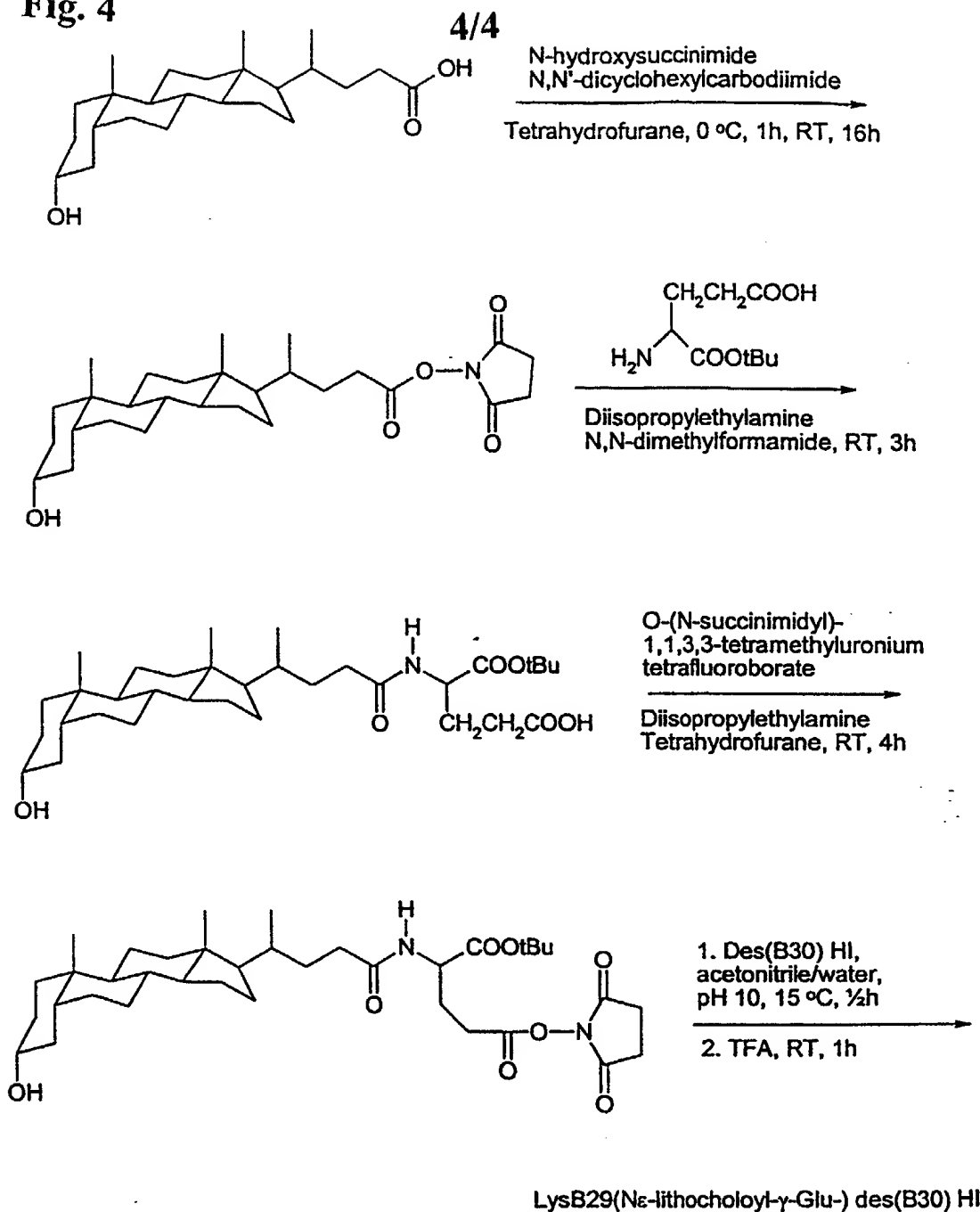




Fig. 4



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 98/00461

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/62, A61K 38/28

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, WPI, TXTE, MEDLINE, SCISEARCH, EMBASE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Proc. Natl. Acad. Sci., Volume 88, November 1991, Victoria Sluzky et al, "Kinetics of insulin aggregation in aqueous solutions upon agitation in the presence of hydrophobic surfaces" page 9377 - page 9381 --	1-11,32-44
A	WO 9731022 A1 (NOVO NORDISK A/S), 28 August 1997 (28.08.97) --	1-44
A	WO 9604307 A1 (ELI LILLY AND COMPANY), 15 February 1996 (15.02.96) --	1-44

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

26 January 1999

Date of mailing of the international search report

29 -01- 1999

Name and mailing address of the ISA/

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 98/00461

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>Journal of Pharmaceutical Sciences, Volume 85, No 3, March 1996, Peter Kurtzhals et al, "Albumin Binding and Time Action of Acylated Insulins in Various species" page 304 - page 308</p> <p>-- -----</p>	1-44

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 98/00461

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 43, 44  
because they relate to subject matter not required to be searched by this Authority, namely:  
Although claims 43 and 44 relate to methods of treatment of the human body (see PCT Rule 39.1(iv), a search has been carried out based on the alleged effect of the aggregates.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

21/12/98

International application No.

PCT/DK 98/00461

Patent document cited in search report			Publication date	Patent family member(s)		Publication date
WO	9731022	A1	28/08/97	AU	1766497 A	10/09/97
<hr/>						
WO	9604307	A1	15/02/96	AU	3236895 A	04/03/96
				CA	2196569 A	15/02/96
				EP	0781295 A	02/07/97
				JP	10503523 T	31/03/98
				US	5559094 A	24/09/96
<hr/>						